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PRINCIPAL INVESTIGATOR: Celine Aznar-Derunes, Ph.D.

CONTRACTING ORGANIZATION: The Burnham Institute
La Jolla, California 92037

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Celine Aznar-Derunes, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Burnham Institute
La Jolla, California 92037

E-Mail: cderunes@burnham.org

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13. ABSTRACT (Maximum 200 Words)
Mutations in normal cellular genes are generally linked to human cancer. Multiple gene changes involving at least two types of cancer genes, protooncogenes and tumor suppressor genes, are required for the clonal expansion of malignant cells. The RIZ gene plays an important role in human cancer and more particularly in breast cancer. RIZ is the founding member of the PR-domain family of zinc finger genes. Two protein products are produced from the RIZ gene which differ by the presence or the absence of the PR domain: RIZ1 and RIZ2. RIZ1 is commonly lost or underexpressed in tumors whereas RIZ2 is always present. RIZ1 is a tumor suppressor whereas RIZ2 is not.

In this project, experiments were performed to characterize RIZ1-PR and related PR domains using protein crystallography and other biophysical techniques. Our goal is to understand the role of RIZ1 and PR-proteins in breast cancer to provide new tools for possible treatment in breast cancer.

During these three years, we have successfully engineered and stabilized three PR domains. Two of these protein products are now crystallizable, and represent the first critical step toward structure determination to define the molecular basis for RIZ tumor suppressor activity.

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INTRODUCTION

The retinoblastoma interacting zinc finger (RIZ or PRDM2) gene was isolated originally in a functional screen for proteins that bind to the retinoblastoma (Rb) tumor suppressor (1). RIZ maps to the human chromosome band 1p36.23 (2,3). This region commonly undergoes deletions, rearrangements or loss of heterozygosity in tumors including human mammary cancer (4).

RIZ is the founding member of the PR-domain family zinc finger genes. It contains an Rb-binding motif called the AR domain similar to the E1A viral oncoprotein (5), eight zinc finger motifs, some GTPase and SH3 domains and a PR domain at its N-terminus (6, 7, 8, 9). The RIZ gene normally produces two different protein products, RIZ1 and RIZ2, which respectively differ in length by the presence or the absence of the PR domain. RIZ2 is produced by an internal promoter (7). RIZ1 is a tumor suppressor whereas RIZ2 is not. RIZ1 expression is commonly lost in human breast cancer tumors as well as in several other types of tumors examined (9), or missense mutations in RIZ1 in these same cancers occur within or near the PR domain (10). In contrast, RIZ2 is uniformly expressed in all cases examined. So there may be a specific negative selection for RIZ1 versus RIZ2 in tumors, called a "Yin-yang " balance.

We are focusing on the PR domain of RIZ1 which plays a role in tumorigenesis in breast cancer. The PR domain is composed of 118 amino-acids and is conserved in a subfamilly of kruppel-like zinc finger genes. Other members of the PR family include the MDS1-EVI1 breakpoint gene involved in human leukemia and the PRDI-BF1 or BLIMP1 transcription repressor, which can activate B-cell maturation (11-16). For these two genes, two protein products are also expressed that differ in the presence or the absence of an N-terminal PR domain. Different observations suggest that PR genes or the PR-containing product of these genes are the negative regulators of cell growth and tumorigenesis. A common function of the PR domain is to mediate protein-protein interaction. The PR domain of RIZ1 is a protein–binding interface (6). Binding is mediated by residues conserved among different PR domains suggesting that similar functions may be shared among them.

The PR domain was found to be homologous to the SET domains (6) (they bear 20-30% sequence identity) which have recently been shown to mediate protein-protein interaction (17, 18). PR and SET domains induce a methylation of the histone 3 (19-20) and define a new class of tumor suppressor genes (21, 22). Furthermore, PR proteins may function in chromatin-mediated control of gene expression as inferred from their homology with SET proteins (6).

The three-dimensional structures of several SET domains have already been determined by x-ray crystallography (23-29). In contrast, there is no structural information available for the PR domains. Our long term goal was then to determine on one hand the crystal structure of the RIZ1-PR domain and on the other hand the crystal

structure of some related PR domains: the BLIMP-PR domain called BLPR and one part of the entire RIZ1 protein containing the PR and AR domains. Doing this, we could understand the tumor-suppression mechanism of RIZ1 and PR-proteins in breast cancer.

During these three years, we used successfully some biochemical and biophysical techniques to define stable and crystallizable constructs of these three proteins.

BODY

Task 1 : To construct a stable RIZ PR domain.

- *Computer modeling/threading*

As the PR domain shares sequence homology with SET domains (Appendix 1), we tried to build an homology model of our RIZ1-PR domain based on the different structures of SET domains. But the sequence similarity between PR and SET domains is 20-30% amino-acid identity and this percentage corresponds to the limit for a homology model validation. In addition, the SET domain does not contain extensive secondary structure as a template model. Homology modeling of turns and loops is challenging and the presence of intervening segments with broad sequence variability makes structural prediction difficult. That is why we did not use a PR model to find the best crystallizable construct of RIZ1-PR.

- *DXMS (Deuterium exchange Mass Spectroscopy)*

We used this new technique with the help of our collaborator Dr Virgil Woods from UCSD to enhance the prospects for crystallization and future structural studies (30-31). This technique helped us to trim the terminal regions of the RIZ-PR domain that we expect may be misfolded or flexible, to produce a more compact domain.

Deuterium exchange maps were generated for the RIZ-PR domain employing deuterium exchange conditions (10 sec and 0 °C) that selectively deuterate highly solvated regions of proteins. On the maps, it can be seen that the N-terminal sequence was highly deuterated, suggesting that this segment is exposed to solvent and may be flexible. So, a new construct was designed to encode protein that was truncated at the N-terminus, removing the flexible segment from the expressed protein product (construct in Appendix 2).

- *Protein expression and purification*

All the constructs (Appendix 2) were engineered in the lab of Dr Shi Huang (The Burnham Institute).
The RIZ-PR domain and the N-terminally truncated one were expressed as GST-fusion proteins. The first step of purification was done by affinity chromatography on a glutathione-agarose (GA) column. After a thrombin cleavage, the proteins were separated from cleaved GST and thrombin by a chromatographic step using tandem para-benzamidine/GA columns. A gel filtration column achieved the purification steps.
At the end we have obtained milligrams of really pure proteins to initiate crystallization trials.

Task 2 : To crystallize the RIZ PR domain.

Several conditions were tested in the first crystallization trials: in our lab by using pre-made crystallization kits from Hampton-Research (Crystal screens I and II) and Emerald Biostructure (Wizard I and II) or our own crystallization systems (changing temperature, concentration...) and in the Hauptmann-Woodward facility for high throughput screening (Buffalo, NY).
These crystallization trials were done on the RIZ-PR domain alone, or in presence of co-factors : the S-adenosylmethionine (SAM) and the S-adenosylhomocysteine (AdoHcy) (17-22). Up to this time we have not be able to detect any interesting crystals.

- *Crystallization trials on the new RIZ-PR domain construct (without N-terminus):*

This new shortened protein, lacking potentially flexible N-terminal residues, formed needles under conditions where its longer counterpart failed to form crystals. The conditions that produced needles are now being optimized by seeding methods to produce large diffraction-quality crystals.

- *NMR experiments:*

The length of this construct (161 amino-acids) allowed us to test the protein in solution by NMR experiments. The preliminary spectra revealed that this domain, which is shortened at the C-terminus is soluble and contains well-ordered secondary structure. Our colleague Dr Klara Briknarova is continuing these experiments to determine the solution structure of this PR domain by NMR.

Task 3 : To solve the crystal structure of RIZ PR domain.

This part depends on the production of diffraction quality crystals from task 2, so we did not begin this part.

Task 4 : To solve the crystal structure of related PR domains.

a) To construct stable proteins

- *Mutations and protease cleavages*

  - BLPR:

The PR domain of BLIMP has been made as a GST-fusion protein. The only way to have a stable construct of this protein is to use a thermolysin cleavage after purification which trims the last 21 amino-acid from the C-terminus (Appendix 2).

- RIZ1

We encountered difficulties during the RIZ1 protein purification because of the degradation of the protein during the process. We determined by mass spectroscopy and amino-acid analysis that the degradation problem occurred between the PR and the AR domains of RIZ. After the mutation of the only arginine present in this connecting segment, (arginine 160) the mutant protein product is more stable. But to really stabilize the protein for weeks at 4°C, experiments were required to finish the purification steps by including an unfolding/refolding step.

- *DXMS (Deuterium exchange Mass Spectrosopy)*

Deuterium exchange maps were generated for BLPR and RIZ1. Deuterium exchange was then performed employing conditions (10 sec and 0 °C) that selectively deuterate highly solvated regions of proteins. On the maps, it can be seen, like for the RIZ-PR domain, that the N-terminal sequence was highly deuterated, indicating that this segment in the domain is flexible or disordered. A new construct for BLPR was designed to encode protein that was truncated at the N-terminus (Appendix 2).

- *Protein expression and purification*

- BLPR:

The BLPR protein and the N-terminally truncated one were expressed as GST-fusion proteins. The first step of purification was done by affinity chromatography on a GA column. For the truncated BLPR an extra step was added before thrombin digestion, i.e., an hydrophobic interaction chromatography (phenyl-sepharose column). After a thrombin digestion, the proteins were separated from cleaved GST and thrombin by a chromatographic step using tandem para-benzamidine/GA columns. The purified BLPR domain was then proteolytically trimmed with thermolysin. A gel filtration column achieved the purification steps for the two proteins.

At the end we have obtained milligrams of really pure protein in the two cases.

- RIZ1

The GST-RIZ1 protein was purified by affinity chromatography (GA column), and thrombin cleavage followed by ion-exchange chromatography (Q-Sepharose column) to remove the GST-partner and then by hydrophobic interaction chromatography (Phenyl-Sepharose column). A gel filtration (Superdex-75 column) was required to remove traces of protease. The protein was then unfolded in 8M urea in the presence of 2mM EDTA and refolded by decreasing step by step the quantity of urea (4M, 2M and 0M urea) in the presence of 2mM $MgCl_2$. Finally, an ion-exchange chromatography (Q-Sepharose column) was used to separate non-folded protein from the refolded protein.

This protocol gave us a good amount of protein to begin the crystallization trials.

b) Crystallization trials

- BLPR

The PR domain of BLIMP and the truncated-BLPR were introduced into crystallization trials with and without the two co-factors SAM and AdoHcy. Screens were performed with the Hampton Research reagents and the Emerald Biostructure kits and more particularly the conditions which contain a high salt concentration which were used for homologous SET domains. Samples were also sent to the Hauptmann-Wooward high-throughput screening facility.

We obtained better needles with the truncated-BLPR than with the full BLPR PR domain. These conditions are now being optimized by seeding methods to produce large diffraction-quality crystals.

- RIZ1

We tried to crystallize the protein by using the Hampton screening reagents and the Emerald Biostructure kits. Samples were also tested at the high throughput screening facility. All the crystallization trials were done at 4°C. No suitable crystals were obtained in these conditions. We expect that the new construct of the entire RIZ1 obtained after trimming of the N-terminus part will give better results, like for RIZ-PR and BLPR.

KEY RESEARCH ACCOMPLISHMENTS

- Overexpression, purification and stabilization of the RIZ1 and BLPR proteins
- Preparation of a stable RIZ-PR domain sample for NMR experiments
- Determination of crystallizable constructs of RIZ-PR domain and BLPR
- Production of a first set of crystals of the truncated RIZ-PR and BLPR protein products.

REPORTABLE OUTCOMES

- Publication in press: Derunes C., Briknarova K., Geng L., Li S., Gessner C.R., Hewitt K., Wu S.D, Huang S., Woods V. I., Ely K. R. Characterization of the PR domain of RIZ1 histone methyltransferase. Biochemical and Biophysical Research Communications.
- Meeting: The 18th Symposium of the Protein Society, San Diego, California, 14-18 August 2004.
- Employment: I have been hired as an Analytical Scientist by a San Diego Biotech Company, Halozyme Therapeutics, for developing purification and characterization of human recombinant enzymes.

CONCLUSIONS

During these three years, we have successfully engineered and stabilized three proteins containing PR domains belonging to the same family of protein: The RIZ-PR domain (161 amino-acids), the BLPR (181 amino-acids) and the RIZ1 protein (325 amino acids). To do that we used different biochemical techniques and a new useful technique called DXMS. These proteins were introduced to crystallization trials which will allow us to determine their 3D structures.
The new construct of RIZ1-PR domain gave encouraging crystals (needles) and the old construct is being used in NMR studies.
The new construct of the BLPR domain also gave preliminary crystals which need to be seeded in order to obtain suitable crystals for the crystallography studies.
We are now designing a new construct of the RIZ1 domain which hopefully will lead to success (like in the case of the two other proteins) in its structural studies by crystallography.

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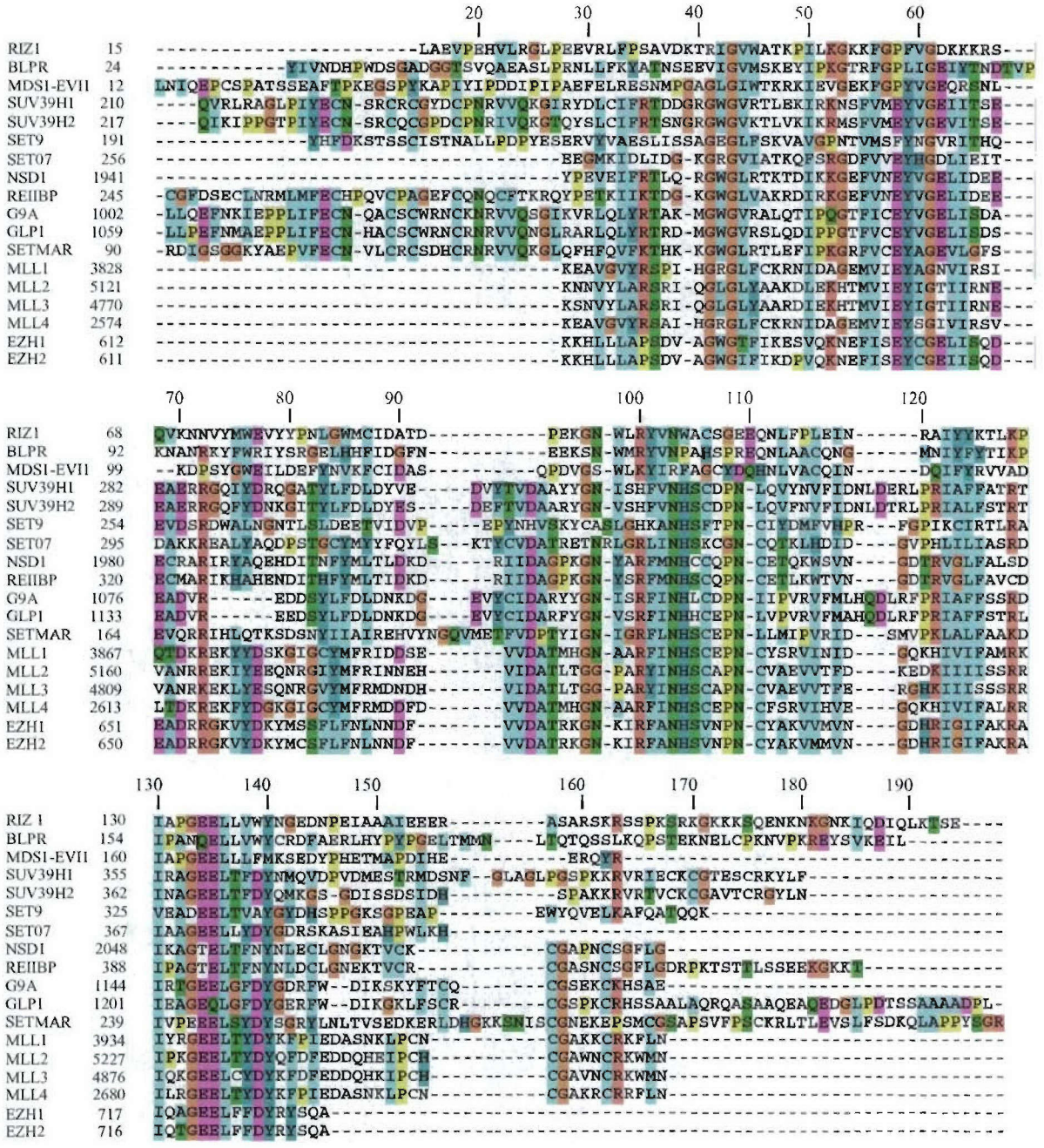
APPENDIX 1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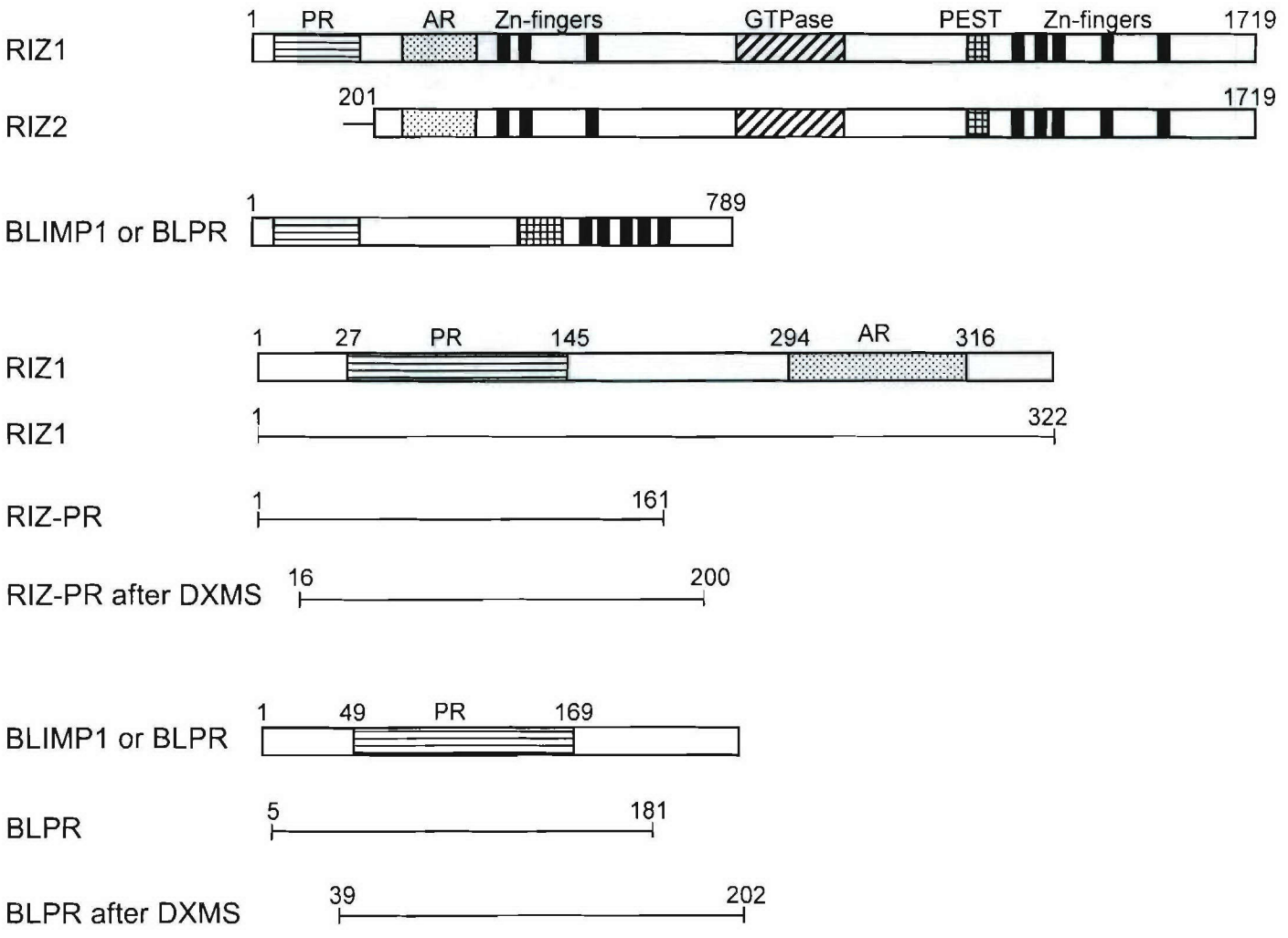
APPENDIX 2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